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METABOLISM OF [^{14}C]GA_{19} AND [^{14}C]GA_{53} BY ECOTYPES OF *BETULA PENDULA*: THE ROLE OF PHOTOPERIOD

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ABSTRACT: In many temperate woody species, short days (SDs) induced growth cessation has been shown to be prevented by the application of gibberellins (GAs). Evidence has also been forwarded for the influence of photoperiod on the metabolism of applied GAs. Continuing with this line of research, we studied the metabolism of ^{14}C-labelled GA_{19} and GA_{53}. [^{14}C]GA_{19} and [^{14}C]GA_{53} were applied to the apices of the northern ecotype (67° N) and to the leaves of the southern ecotype (64° N) of *Betula pendula* Roth. under different photoperiods and at different times in order to compare metabolism during day and night periods. Assay of metabolites was made 10 h after application. Less than half of each applied GA was taken in by the plants and most of the activity was recovered from the application points. There was no clear effect of photoperiod on uptake of the GAs. After the HPLC analysis, most of the absorbed radiolabelled GAs were eluted as unmetabolised forms. However, significant levels of gibberellins A_{20}, A_{29}, A_1, and A_8 were recovered as metabolites of [^{14}C]GA_{19} both in apical stems and leaves. In 1SD and 4SD-treated plants, there was less metabolism of [^{14}C]GA_{19} compared to LD-treated plants. The difference was due to reduced metabolism of [^{14}C]GA_{19} by SD-treated plants during the night period. On the other hand, metabolism of [^{14}C]GA_{53} was very limited in all day-length treatments and during both, day and night periods. The most abundant metabolite was [^{14}C]GA_{19} (10% of the activity in the samples).

Key words/phrases: Conjugation, ecotype, gibberellins, metabolism, radioactivity

Abbreviations: [^{14}C]GA_x, radioactive carbon labelled gibberellin x; LD, long day; SD, Short day; 1SD, one short day; 4SD, four short days; HPLC, higher performance liquid chromatography; i.d., internal diameter; PVPP, polyvinylpolypyrolidone; PAR, photosynthetically active radiation

INTRODUCTION

The influence of light on the metabolism of labelled GAs has been shown in several experiments (Musgrave and Kende, 1970; Bown *et al*., 1975; Rood *et al*., 1986). When spinach (*Spinacia oleracea*) plants were transferred from short days (SDs) to long days (LDs), the level of endogenous GA_{19} decreased while the levels of GA_{20} and GA_{29} increased (Metzeger and Zeevaart, 1980). In further studies on this system, Gianfagna *et al*. (1983) showed that [^{2}H]GA_{53} applied to spinach leaves was converted to [^{2}H]GA_{20} in LD, but only to [^{2}H]GA_{44} and [^{2}H]GA_{19} in SD. This was shown to be a result of increased activity of enzymes converting GA_{53} and GA_{19} to GA_{44} and GA_{20}, respectively, in LD compared to SD (Gilmour *et al*., 1986). On the other hand, the enzyme oxidising GA_{44} remained high irrespective of light or dark treatments (Gilmour *et al*., 1986).

Most of the studies on GA metabolism have focused on biosynthesis of the free acids. Howover, GA conjugation is a common process especially when rate of metabolism exceeds the required quantity of free GAs (Schneider and Schliemann, 1994). The term conjugated GAs refer to compounds which are formed by covalent coupling of GAs to other low molecular weight substances, mostly glucose, while the term bound GAs is restricted to GAs associated with macromolecules, proteins and cell walls (Schneider and Schliemann, 1994; Graebe, 1987). Most common GA conjugates are those attached to glucose, and it is also possible that they can be decomposed to release free GAs (Rood *et al*., 1986; Schneider and Schliemann, 1994; Graebe. 1987; Schneider *et al*., 1999). There is very little information about the possible effects of photoperiod on conjugation of GAs.

Although only few GAs have been conclusively identified from woody plants, GA_1 appears to be the bioactive GA in species like *Salix pentandra* L., *Betula sp.* and *Alnus sp.* (Junttila, 1991, Junttila *et al.*, 1991). The early 13-hydroxylation pathway is the main biosynthetic pathway in many hardwoods (J. E. Olsen, personal communication), and members of this pathway have been identified also in *B. pendula* Roth. (Zanewich and Rood, 1994). GA_{19} often dominate quantitatively in these species and it is the pool for the bioactive GA. Application of exogenous GA_1 to *B. pendula* ecotypes under non-inductive photoperiod can substitute the effect of LD, while GA_{20}, although it induces growth, is less active (Berhanu A. Tsegay, 2002). However, GA_{19} is primarily ineffective. Similar results have been obtained with *B. pubescens* (Junttila, 1993). In *S. pentandra* (Junttila and Jensen, 1988) and *Populus sp.* (Mølmann *et al.*, 2003), GA_{20} could also substitute the effect of LDs. These application studies, as well as analyses of endogenous GAs in *S. pentandra* (Olsen *et al.*, 1995a; 1997a and b) and in *B. pendula* (Berhanu A. Tsegay, 2002), indicate that biosynthesis of GA_1 could be controlled by photoperiod. Effects of photoperiod on metabolism of GAs have been studied in *S. pentandra* (Olsen *et al.*, 1995b), but so far only minor effects have been reported. However, in those metabolism studies (Olsen *et al.*, 1995b), a dihydro-GA_{19} was used as a labelled GA_{19}, and it is now known that dihydro-GAs are significantly less active than the corresponding normal GAs (Olsen and Junttila, 1997). Hence, the purpose of this experiment was to re-examine the effect of photoperiod on the metabolism of GA_{19} and GA_{53} in *B. pendula* using ^{14}C labelled compounds. Since GA metabolism had previously not been investigated in different ecotypes, metabolism of [^{14}C]GA_{19} and [^{14}C]GA_{53} was also studied in contrasting ecotypes of *B. pendula*. Moreover, GA metabolism during the day and during the night has not been studied before.

MATERIALS AND METHODS

Plant material and cultivation conditions

This experiment was conducted in the phytotron, the University of Tromsø. Seedlings of two latitudinal ecotypes of *B. pendula* Roth were used. Seeds were collected from the middle of Norway (64° N, southern ecotype) and northern Norway (67° N, northern ecotype). They were stratified in moist sand for about a month at 0.5°C and germinated in continuous light (180–200 µmol m^{-2} s^{-1}) from fluorescent tubes (Philips TLD 58 / 840) and incandescent bulbs at 18°C. After germination, seedlings were transferred to 21°C with similar light treatments. Eight days later, they were potted in 12 cm diameter pots, 5 plants per pot, in a mixture of fertilised peat: perilite: and sand (6:3:1). There were 2 pots per parallel. Plants were watered daily. When the plants reached the height of about 5 cm, they were transferred to their respective treatments. During the treatments, plants were grown at 18°C either in LD (12 h PAR extended by 12 h incandescent bulbs), or SD (12 h PAR and 12 h dark) conditions.

Application of GAs

^{14}C labelled GA_{19} and GA_{53} (specific activity of 2.072 X 10^6 Bq.µmol^{-1} for both) were obtained from Prof. L. Mander, Australian National University, Australia. Two sets of experiments were done one with an application as microdrops to the apices, the other by injecting to the dorsal surface of the upper-most fully expanded leaves. About 10.6 MBq [^{14}C]GA_{19} and [^{14}C]GA_{53} per plant was applied in 1 µl 96% ethanol to the apex of the northern ecotype, while about 11.6 MBq per plant was applied by injection to the dorsal surface of the uppermost fully expanded leave of the southern ecotype.

For each ecotype day-time and night-time metabolism of [^{14}C]GA_{19} and [^{14}C]GA_{53} were studied. For studies of the day-time metabolism, ^{14}C labelled GAs were applied at the start of the PAR light period under LDs or after one short day (1SD) and four short days (4SDs). For studies of the night-time metabolism, application was done at the start of the light extension period under LDs or at the start of the dark period after 1SD and 4 SDs (under green safe light). In all cases, plants were harvested 10 h after application with 2 replicate samples, each consisting of 10 plants.

Harvest of samples

About 15 mm of the upper part of the shoots were collected and washed for 1 minute in 10 ml dist. H_2O, and then surface dried with tissue paper. Apically applied plants were then divided into three samples: shoot tips that consisted of about 5 mm of the apical stem with the youngest unfolded leaves, stems that consisted of about 10 mm of the stem below the apex, and leaves on this stem piece. For plants where leaf injections had been done, the samples collected were shoot tips and stems as above, applied leaves, and leaves above the applied leaf. The samples were stored at –20°C in vials with 5–10 ml 80% methanol.

Extraction and purification of GAs

Radioactivity recovered during washing was counted. Plant samples were homogenised with an ultra-thurrax and extracted for about 20 h at 4°C. The residue was removed by filtering. The extract was reduced to the water phase using rotavapour, 40°C. Five milli liter buffer, pH 8, and some washed polyvinylpolypyrolidone (PVPP) (about 1 g for every 10 ml solution) was added, frozen over night, thawed and filtered. After adjusting pH to 2.5, the extract was partitioned 3 times against equal volumes of ethyl acetate. Aliquots of the ethyl acetate and the water phases were taken for counting of radioactivity. The water phase was discarded. The ethyl acetate was evaporated to dryness (40°C). The residue was dissolved in a small amount of methanol and about 5 ml water, adjusted to pH 8 and applied to a 5 x 1 cm i.d. QAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) anion exchange column. In advance the column was equilibrated and washed with about 40 ml water at pH 8. The GAs were eluted with 50 ml 0.2 M formic acid directly on to a pre-equilibrated 0.5 g Sep-Pak Vac C_{18} cartridge (Waters Associates, Milford, MA, USA). GAs were eluted from the C_{18} column with 4 ml 80% methanol and the solvent was evaporated in a speedvac.

HPLC and scintillation analysis

The samples were dissolved in 50 µl of 50% methanol. They were purified by reversed phase HPLC using standard instruments from Waters (Waters Associates, Milford, *MA, USA*). A 100 x 8 mm i.d. novac-pak C_{18} column was operated at 25°C (for details, see Olsen *et al*., 1994; Junttila *et al*., 1997). The mobile phase was a linear gradient of 25 min from 20 to 80% acidified methanol in acidified water (1% aqueous acetic acid) at a flow rate of 2 ml min^{-1} (25°C). Fractions were collected at 1 min intervals. After addition of a scintillant (Ultima Gold XR Packard, Canberra) to the samples, radiocounting was done with a Tri-Carb 2300 TR liquid Scintillation Analyser.

RESULTS AND DISCUSSION

Uptake of [^{14}C]GA_{19}

Most of the radioactivity applied to both, the apices and the leaves, was removed during washing. When the distribution of radioactivity taken up by the plant was determined 10 h after application of [14C]GA_{19} , most of the labelled GA (87–96%) was recovered at the application points: apices for the northern and leaves for the southern ecotype (Table 1). Thus, translocation of radioactivity was limited. The lowest amounts of radiocounts recovered, on average, were in stems (1.3%) and in apices (1.9%) for the northern and the southern ecotype, respectively, during day metabolism (Table 1). Although the percentage of uptake varied between applications, no obvious influence of photoperiod was observed.

Table 1. Distribution of radioactivity in homogenized tissues of different plant parts of two ecotypes of *B. pendula* under long (LD) and short (SD) days. Mean± range is indicated.

Ecotype	Treatment time	Treatment group	Total activity recovered (MBq)	Recovered radioactivity (%)			
				Applied leaves	Apex	Young leaves	Stem
Northern	Day	LD	49.9±3.3		90.5	6.5	3.0
(67? N)		1SD	27.8*		95.9	3.5	0.6
		4SD	48.2±4.5		94.4	5.4	0.2
		Mean	*41.9*		*93.6*	*5.1*	*1.3*
	Night	LD	53.5±14.1		87.4	4.2	8.4
		1SD	70.7±1.6		95.2	4.5	0.3
		4SD	19.2±2.8		86.9	12.6	0.4
		mean	*47.8±6*		*89.8*	*7.1*	*3.1*
Southern	Day	LD	50±0.6	90.6	3.3	2.8	3.2
(64? N)		1SD	59.7±2.1	91.7	1.4	2.5	4.4
		4SD	26.9±1.8	96.0	1.1	2.1	0.8
		mean	*45.9±1.5*	*92.8*	*1.9*	*2.5*	*2.8*
	Night	LD	65±4.5	88.2	9.6	0.7	1.4
		1SD	64.2±0.1	87.8	4.2	2.9	5.0
		4SD	23.2±2.5	93.0	2.8	2.9	1.3
		mean	*50.8±2.3*	*89.7*	*5.6*	*2.2*	*2.6*

* One sample

Putative conjugation

After purifying the extracts with PVPP, the aliquot was partitioned against ethyl acetate at pH 2.5. Under these conditions, most of the free GAs are extracted into the organic phase, and most of the putative conjugates remain in the aqueous phase. Large part of the radioactivity (about 82 to 84% on average) passed to the organic phase. The amount of conjugation (on average) was relatively low in leaves of both ecotypes of *B. pendula*, ranging from 4.9 to 9.5%, while in the stems and apices it ranged from 19.2 to 23.8% (Table 2). There was no effect of SD treatment on the formation of conjugates, and the amounts of conjugates formed during the day and night periods were very similar.

Metabolism of [^{14}C]GA$_{19}$

Ethyl acetate fractions were subjected to reversed phase HPLC in order to obtain a profile of the metabolites, which were tentatively identified based on co-chromatography with standards. In general, chromatograms were similar for the various tissues analysed.

Analysis by HPLC of the apical tissue of the northern ecotype showed a similar pattern of main peaks of radioactivity in all treatments (LD, 1SD and 4SD) and application times (day and night). Large percentage of recovered radioactivity was due to unmetabolized [^{14}C]GA$_{19}$ (Table 3). There was a slight indication that plants in SD metabolised less of the applied [^{14}C]GA$_{19}$ during the night than during the day (Table 3).

Table 2. Putative conjugation of [^{14}C]GA$_{19}$ expressed as per cent radioactivity recovered in the aqueous phase after partitioning with ethyl acetate. Total counts for the samples are given in MBq.

Ecotype	Time	Plant part	Treatments						Mean±SE aqueous phase (%)
			ld		1sd		4sd		
			Total count	aqueous phase (%)	Total count	aqueous phase (%)	Total count	aqueous phase (%)	
Northern	Day	Apex	41.1	20.6	26.9	20.4	30.1	16.7	19.2±1.3
		Leaves	6.3	7.5	0.3	6.7	1.9	10.4	8.2±1.1
		Stem	2.5	18.3	0.6	25.0	0.2	21.1	21.5±1.9
		mean	16.6	15.3	10.2	17.4	10.8	16.0	16.2±1.1
	Night	Apex	37.1	18.6	60.1	21.8	16.3	18.9	19.8±1.0
		Leaves	8.3	9.9	9.6	10.3	2.4	7.5	9.2±0.9
		Stem	8.1	23.5	1.0	20.7	0.5	27.2	23.8±1.9
		Mean	17.8	17.3	23.6	17.6	6.4	17.9	17.6±0.3
Southern	Day	Applied leaves	39.5	4.4	57.9	5.7	26.3	5.0	5.0±0.4
		Apex	0.1	21.3	0.1	20.6	0.04	18.3	20.1±0.9
		Leaves	1.1	10.1	1.2	9.9	0.5	8.4	9.5±0.5
		Stem	0.3	32.5	0.5	20.7	0.1	13.6	22.3±5.5
		mean	10.3	21.3	14.9	17.1	6.7	13.4	17.3±4.0
	Night	Applied leaves	61.9	5.1	62.4	4.5	22.8	5.2	4.9±0.2
		Apex	0.9	13.3	0.2	20.4	0.0	19.3	17.7±2.2
		Leaves	1.6	8.8	1.0	9.7	0.4	7.6	8.7±0.6
		Stem	0.6	21.8	0.6	18.1	0.0	22.0	20.6±1.3
		mean	16.3	14.6	16.1	16.1	1.0	16.3	15.7±0.9

Table 3. Effect of photoperiod on conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20}, [^{14}C]GA_{29}, [^{14}C]GA_1, and [^{14}C]GA_8 by the apical tissue of the northern ecotype of *B. pendula*. Results were calculated as per cent radioactivity of the total activity in the chromatogram. Mean ± range shown.

Metabolite	HPLC Fraction	% Conversion (100% = KBq/ HPLC fractions included)						Total mean
		LD		1SD		4SD		
		day	night	day*	night	day	night	
	100%=	1140.8±192	1942.8±387	1736	2290.6±274	2390.4±541	1482.2±104	1830
[^{14}C]-GA_{19}	19–21	32.0±3.6	33.0±4.6	31.9	40.0±4.1	33.5±0.8	41±1.4	35.2
[^{14}C]-GA_{20}	15–16	15.3±0.8	9.5±5.6	10.0	11.4±2.2	9.3±1.2	10.7±0.7	11.0
[^{14}C]-GA_{29}	10–13	10.9±4.7	9.4±1.6	10.3	10.4±1.4	12.6±4.2	9.4±0.8	14.7
[^{14}C]-GA_1	10–13	17.0±4.7	14.6±1.6	11.3	11.4±1.4	13.8±4.2	10.3±0.8	8.8
[^{14}C]-GA_8	7–9	8.3±0.6	12.5±3.6	13.2	8.6±0.6	11.7±0.7	6.0±0.4	10.0

*** Single sample**

There was no difference in metabolism of [^{14}C]GA_{19} by apical tissue during the day regardless of the day length. However, there was small variation in the amount of metabolites co-eluted with authentic GAs: GA_8, GA_1, GA_{29}, GA_{20} and GA_{19}. In LD, more labelled [^{14}C]GA_{20}, [^{14}C]GA_{29} and [^{14}C]GA_1 were found when the application was made during the day than during the night (Table 3). Radioactivity corresponding to [^{14}C]GA_8 was, however, greater when the application was at night in LD plants (8% during the day and 13% during the night) (Table 3).

In plants treated with one short day (1SD), radioactivity corresponding to [^{14}C]GA_8 was higher during the day while there was no difference in activities due to [^{14}C]GA_1, [^{14}C]GA_{29} and [^{14}C]GA_{20}. After apex application, radioactivity in the chromatographed samples of leaves and stem tissues was too low to allow for conclusions.

In the experiment with leaf application, metabolism of [^{14}C]GA_{19} in the applied leaf was comparable to that in apex described above, but the difference between day and night period was even clearer, particularly in SD treatments (Table 4). Metabolism of [^{14}C]GA_{19} during night in plants treated with 1SD and 4SD was very small, about 60% of the applied GA co-eluted with GA_{19} at fractions 19–21. Moreover, in these samples, there was only low level of radioactivity in GA_1-fractions and almost nothing in GA_8-fractions.

Radioactivity in the apex samples, after an application to a mature leaf, was too low to produce reliable chromatograms (data not presented). Some radioactivity corresponding to [^{14}C]GA_{19} was found in all treatments and in all samples analysed. This could indicate that even [^{14}C]GA_{19} was translocated in these *B. pendula* seedlings. In stems, much of the radioactivity was in forms other than the GAs of interest after metabolism during the night period. Most of the radioactivity (up to 77% of the radioactivity in the sample) eluted in earlier fractions (1 – 8) implicating them of being polar conjugates. These compounds were not found in the other samples. In this experiment, chromatograms for leaf and apex tissues were rather similar.

Metabolism of [^{14}C]GA_{53}

Metabolism of [^{14}C]GA_{53} was very limited in all the treatments, unmetabolised substrate ranged from 52 to 63%. No difference was observed between the studied treatments or metabolic periods (day–night) (Fig.1). [^{14}C]GA_{19} was the most abundant metabolite, about 10% of the radioactivity in the sample. In addition, radioactivity was detected in fractions corresponding to the elution patterns of GA_{20}, GA_{29}, GA_1, and GA_8.

Table 4. Effect of photoperiod on conversion of [^{14}C]GA$_{19}$ to [^{14}C]GA$_{20}$, [^{14}C]GA$_{29}$, [^{14}C]GA$_1$, and [^{14}C]GA$_8$ by the upper-most matured leaf of the southern ecotype of *B. pendula*. Otherwise as in Table 3.

Metabolite	HPLC Frac.	% Conversion (100% = KBq/ HPLC fractions included) LD day	LD night	1SD day	1SD night	4SD day	4SD night
	100%=	1450±545	9414±561	3620±572	9866±113	3954±561	7340±39
[^{14}C]-GA$_{19}$	19–21	34.4±2.9	36.2±0.9	40.0±0.7	56.0±0.0	37.9±0.5	62.0±0.4
[^{14}C]-GA$_{20}$	15–16	13.0±3.5	17.2±0.1	14.9±2.4	7.6±0.0	15.0±2.5	7.4±0.2
[^{14}C]-GA$_{29}$	10–13	11.8±2.1	8.6±0.5	9.3±1.4	4.6±0.0	8.9±1.4	4.1±0.2
[^{14}C]-GA$_1$	10–13	13.1±2.1	9.5±0.5	7.3±1.4	3.7±0.0	7.0±1.4	3.2±0.2
[^{14}C]-GA$_8$	7–9	8.0±1.3	5.0±0.3	4.7±0.5	2.8±0.2	5.3±0.1	1.8±0.4

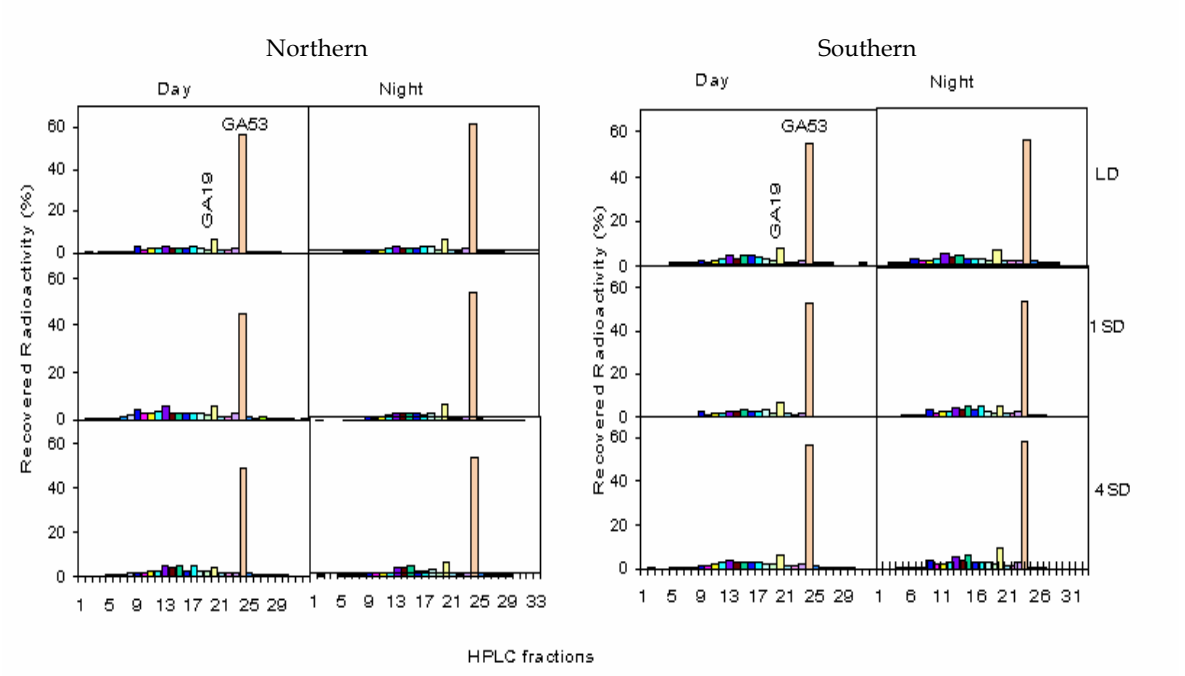

Fig. 1. Metabolism of [^{14}C]GA$_{53}$ by apical tissue of the northern and by leaves (applied leaves) of the southern ecotype of *B. pendula* at different times of the day (day and night) and at different day-length treatments (LD, 1SD and 4SD).

CONCLUSION

Due to a lack of conclusive identification of the metabolites, conclusions from feeding studies are limited. However, it was shown that [^{14}C]GA$_{19}$ was metabolized to [^{14}C]GA$_{20}$, [^{14}C]GA$_{29}$, [^{14}C]GA$_1$, and [^{14}C]GA$_8$ by apical stem tissues and leaves of *B. pendula* ecotypes irrespective of the mode and time of application and the day-length pretreatment (Tables 3 and 4). Likewise, after application of [^{14}C]GA$_{53}$, about 10% [^{14}C]GA$_{19}$ and trace amounts of [^{14}C]GA$_{20}$, [^{14}C]GA$_{29}$, [^{14}C]GA$_1$, and [^{14}C]GA$_8$ co-eluted on reversed phase HPLC with their respective authentic GAs in both ecotypes (Fig 1).

There was more metabolism of [^{14}C]GA$_{19}$ when application was during the day than when it was during the night in SD treated plants (Tables 3 and 4). On the other hand, there was no difference in the metabolism of [^{14}C]GA$_{53}$ between treatments (day-lengths) or applications (day/night) (Fig. 1).

Hence, it appears that photoperiod had an influence on the metabolism of [^{14}C]GA_{19} but not on [^{14}C]GA_{53}. In addition to the GA_{19} – GA_{20} step, the metabolism of GA_{20} to GA_1 seems to be influenced by photoperiod. However, further studies on woody plants would be necessary before proper conclusions on the photoperiodic control of GA metabolism can be drawn.

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